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14. ABSTRACT

Disc injury through trauma, vibration loading, or mechanical overload, and the resulting disc degeneration in response to these insults over time are tremendous problems affecting the active and veteran military population. Current treatment options fail to restore disc structure and mechanical function. Our goal in this proposal is to develop methodologies for the engineering and implanting of a functional biologic disc replacement. Significant progress has been made in the last 12 months towards achieving this goal. We have successfully engineered a concentric annulus fibrosus, the functional properties of which improve with culture time. We have shown the dynamic culture further enhances functional matrix deposition. We have shown that a short period of exposure to transforming at a high dose is equal to or better than long term exposure for stem cells cultured in an engineered nucleus pulposus-like hyaluronan hydrogel. We have developed and validated a minimally invasive surgical technique for implantation of our engineered disc. We have successfully performed in implantation of acellular engineered discs. Finally, we have designed and implemented a novel internal fixation device to enhance retention of the engineered disc and stabilize the joint during healing.

15. SUBJECT TERMS
Intervertebral disc degeneration; low back pain; biological disc replacement; preclinical animal model

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Table of Contents

Introduction:

The focus of this project is on restoration of the intervertebral disc through tissue engineering methodologies. Disc injury through trauma, vibration loading, or mechanical overload, and the resulting disc degeneration in response to these insults over time are tremendous problems affecting the active and veteran military population. Neither conservative treatments, such as stretching and exercise, nor surgical options, such as fusion and arthroplasty, restore disc structure or mechanical function. Total disc arthroplasty is relatively new to clinical practice, but will suffer from the same problems as traditional implant materials – wear and the need for eventual replacement. An alternative to these methods involves implantation with a biologic tissue engineered replacement. Such a biologic tissue restoration method would not be subject to wear as occurs with prosthetic devices, and would restore flexibility and motion about that spinal segment. Because the function of the disc is mechanical, it is important to focus upon mechanics in the design of functional tissue engineered constructs (TECs) and to direct the biology (maintenance of phenotype and ECM deposition) towards these mechanical outcomes. Direct biologic restoration of the disc with a TEC that duplicates the mechanical properties of the native tissue and restores range of motion would be an ideal alternative. Our goal in this project is to develop methodologies for the engineering and implantation of a functional biologic disc replacement. **The objective of this proposal is to move to the translational space and towards clinical implementation by creating and implanting a tissue engineered disc-like angle ply structure (DAPS)**,and throughout this development, remain mindful of the fundamental importance of the mechanical function.

During the first year of funding on this project, the PIs have made substantial progress in the Aims. Given the highly collaborative and interdisciplinary activities of this project, a single document detailing all progress is presented, but is uploaded under each of the PIs specific award numbers. The research team has been meeting on a weekly or bi-weekly basis over this time course, and have established major advances in the formation of disc-like angle-ply structures for disc tissue engineering applications.

Body:

Drs. Elliott, Mauck, and Hebela have made marked progress on their project (OR090090) entitled: “Development and Translation of a Tissue-Engineered Disc in a Preclinical Rodent Model”. Below, we provide a listing of the original specific aims of our project, followed by detailed descriptions from several recent abstracts and submitted papers based on these efforts. Our work stems from the translation of the single and bi-layer constructs with tensile properties approaching that of the native annulus fibrosus tissue into full 3D Disc-like Angle Ply Structures (DAPS), inclusive of a hyaluronic acid hydrogel seeded with adult stem cells that can be used to replace the degenerate native disc.

Proposed Aim 1: Create a mesenchymal stem cell (MSC) seeded 3D structural TEC disc from concentric AF constructs surrounding an engineered nucleus pulposus (NP) composed of a hyaluronic acid (HA) hydrogel. Measure the disc structural mechanics in compression and torsion, and the isolated AF and NP substructures in compression following time in culture. Evaluate the molecular, histological, and biochemical properties of these TEC discs as a function of time in culture and with variations in media conditions.

A substantial portion of Aim 1 has been completed. In the first study, we investigated the biochemical and mechanical effects of orbital shaking on the maturation of multi-lamellar disc-like angle-ply structures (DAPS) in order to expedite growth conditions for AF replicates and, more broadly, to optimize design strategies for MSC-laden multi-layered tissues. This work was published in abstract form as: **Functional Enhancement of Disc-Like Angle-Ply Structures via Dynamic Culture**, by Kluge, JA; Martin, JT; Nerurkar, NL; Amaniera, FA; Pampati, RA; Elliott, DM; Mauck R L, at the 2011 Orthopaedic Research Society in Long Beach, California. A full length manuscript on this work is now under construction and is expected to be submitted soon.

Briefly, the focus and findings of this work are as follows. We formed an annulus fibrosus (AF scaffold) by electrospinning aligned PCL fiber mats ~0.3mm thick. 3 mm wide strips were cut 30^{o} from the fiber direction. Strips were seeded with MSCs and pre-cultured for 2 weeks in chemically defined media with 10 ng/mL TGF-β3. Media was changed twice weekly for the duration of the study. At 0 weeks, strips were paired into bilayers with opposing +/-30^{o} fiber orientations and wrapped concentrically to 10mm outer diameter. After 1 week, the cup holding the DAPS construct was removed to allow for free swelling culture with all surfaces exposed. At this baseline (week 0) or 4 weeks after removing from the retaining cups, DAPS were harvested for analysis. Alternatively, after removing from retaining cups, DAPS were maintained on an orbital shaker set to a 2Hz frequency and likewise harvested at 4 weeks. This was done to improve maturation and to simulate fluid exchange that might be expected in vivo with mechanical loading. Samples were reserved for torsion testing (n=3), in which a custom-designed rig, containing a torque cell (5in.-oz. limit) in series with a stepper motor (LabVIEW controlled), was affixed to a standard Model 5542 Instron uniaxial testing device. Samples were compressed to a creep load of 0.02N for 200 seconds, followed immediately by 25% axial compression of the post-creep height. After a 20 minute relaxation period, DAPS were subjected to 10 cycles of torsion at +/- 6° at 0.05Hz and the last load/unload curve reserved for analysis. Raw torque data was normalized to construct geometry to calculate torsional stiffness [kPa] and range of torque [kPa/°]. Following testing, DAPS samples were stored frozen, lyophilized, papain digested and assayed for collagen, GAG, and DNA content. Likewise, media in both shaken and static cultures were periodically reserved during feeding and measured for GAG content using the DMMB assay. Untested samples (n=2) were sectioned and stained with DAPI, Alcian Blue, and Picrosirius Red to visualize cells, proteoglycans, and collagen, respectively. Significance was determined by one-way ANOVA with Tukey's post hoc ($p \leq 0.05$).

In carrying out this work, we found that both statically and dynamically cultured MSC-laden DAPS increased in torsional mechanical properties and biochemical content with time in culture. By 4 weeks, PG staining (Alcian Blue, Fig 1), was significantly improved in both static and dynamic groups, but was more homogenous throughout constructs with dynamic culture. Although there was a significant increase in total GAG in dynamic culture groups compared to their static counterparts (Fig. 2), the GAG measured within the cell culture media surrounding each construct (measured every 3-4 days) accumulated to roughly equal levels as the static culture group. The GAG measured in dynamic constructs was roughly 40-70% that of human annulus tissue, depending on the age and anatomical location within the disc space.

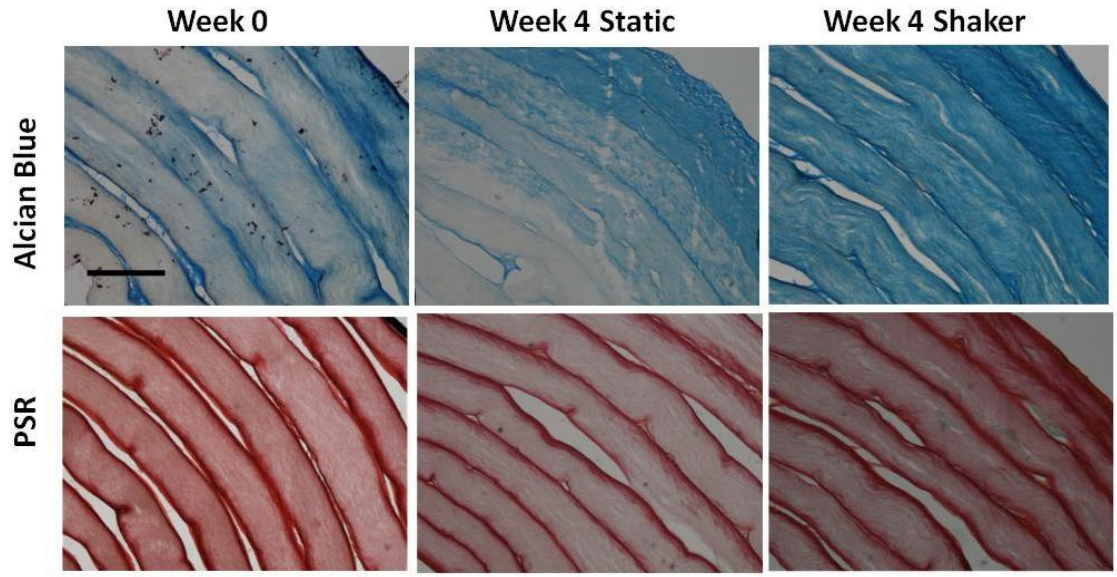

Figure 1. Histology of DAPS samples sectioned throughout the specimen midline and stained with either Alcian Blue or Picrosirius Red (PR). Scale = 500 μm.

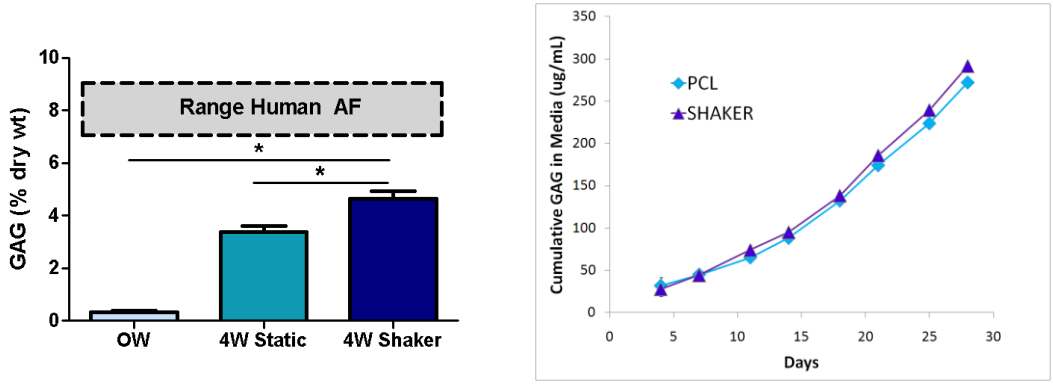

Figure 2. (Left) GAG content, reported in % dry weight (%DW). *=p<0.05 static compared to dynamic groups and both groups at 4 weeks compared to 2 weeks. Dash-lined box indicates native human AF benchmarks. (Right) GAG measured periodically in media for static and dynamic groups over 4 weeks.

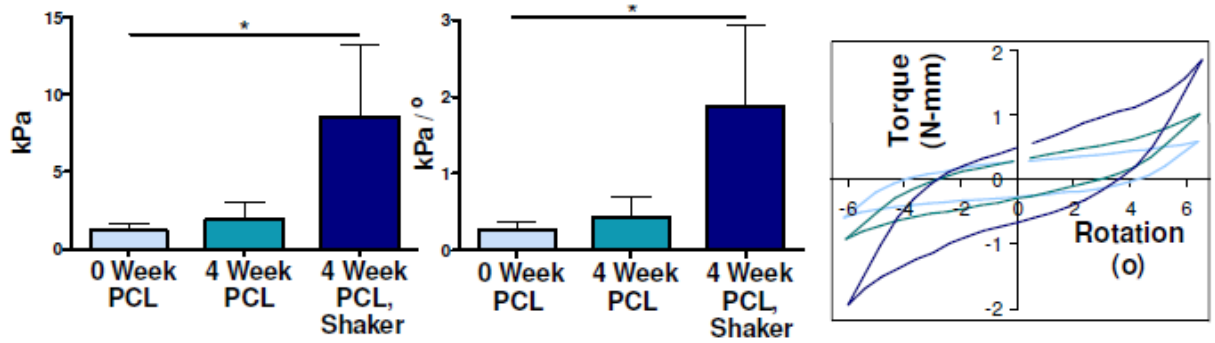

Figure 3. Torsional stiffness (left) and range of torque (center), n=4/group. (Right) Representative rotation-torque response of DAPS with time in culture. * = p≤0.05 compared to 0 week.

This study demonstrated the beneficial effects of dynamic culture on the maturation of MSC-laden DAPS at a length scale of the full intact tissue. This is the first study to our knowledge showing beneficial effects of fluid mixing in the context of multi-lamellar electrospun mats tested using physiologically-relevant strategies (ie torsional mechanics). The increased levels of GAG incorporated across the lamellar space, while maintaining GAG deposits into the media, suggests that GAG produced by these MSCs positively responds to the culture environment and is free to migrate, helping to produce a more mature fibrocartilaginous matrix. However, since our biochemical and mechanical characterization suggests that our engineered disc falls short of several native tissue benchmarks, we will continue to pursue options such as longer culture durations and options to increase the porosity of nanofibrous mats in order to promote enhanced inter-lamellar intra-lamellar organized ECM. Importantly, this work established all methodologies required for the formation and culture of the angle-ply portion of the DAPS. Moreover, we demonstrated biomechanical and biochemical maturation of the AF construct as proposed. The finding that mechanical stimulation (via orbital shaking) improved growth suggests that our implanted DAPS structure might similarly benefit form fluid flow induced by loading after implantation.

In a second recent study in furtherance of the completion of this Aim, we examined the effect of transient exposure to chondrogenic medium to better understand the duration and time course that best establishes the NP and AF cell phenotype. Our focus here was on the formation and maturation of the mesenchymal stem cell (MSC) based nucleus pulposus (NP) region of our DAPS construct. This work was recently published as an abstract at the 2011 ASME Summer Bioengineering Conference in Nemacolin Woods, PA. The title of the abstract was: **Transient exposure to TGF-ß3 improves the functional properties of MSC-seeded photocrosslinked hyaluronic acid hydrogels** by authors Minwook Kim, Isaac E. Erickson, Jason A. Burdick, and Robert L. Mauck. A paper on this topic (with the same title and author list) is now undergoing minor revision for publication in the *Journal of the Mechanical Behavior of Biomedical Materials*.

To carry out this study, MSCs were isolated from tibial bone marrow, expanded in culture through passage 2-3, and seeded (60 million cells/mL) in 1% w/v photocrosslinkable HA (Lifecore Biomedical) as in our preliminary studies. Cylindrical MSC-based NP constructs (Ø4 × 2.25mm) were cultured in a chemically defined medium (1mL/construct). Constructs were then exposed to TGF-β3 at several different concentrations and durations, and compared to continuous exposure of TGF-ß throughout the culture period. Note, these doses and timings were expanded slightly from that proposed to better capture the full range of possible dosing schemes. The TGF-β3 dose was varied from the standard concentration (10ng/mL), to a high dose (50ng/mL), or to a very high dose (100ng/mL). For these higher concentrations, exposure times were limited to 3 or 7 days, with media changed one time over the first week. For the lower dose, exposure was for 3, 7, 21, or 42 days. A total of 8 groups were thus evaluated (10-cont, 10-21d, 10-3d, 10-7d, 50-3d, 50-7d, 100-3d and 100-7d), where the first number indicates dose, and the second number indicates duration. Media was changed twice weekly for the duration of study. Cell viability was assessed with Live/Dead staining (Invitrogen). Unconfined compression testing was carried out to determine construct dynamic and equilibrium properties. Total dsDNA, sulfated glycosaminoglycan (s-GAG), and collagen content was determined after papain digestion. Paraffin embedded sections (8µm) were stained with Alcian Blue for proteoglycan (PG) and Picrosirius Red for collagen. Significance was determined by two-way ANOVA with Tukey's post hoc test ($p<0.05$).

In carrying out this study, we determined that MSCs in our NP regions were viable and biosynthetically active in HA hydrogels under every condition. Constructs were geometrically stable with time (Fig.4A), an important consideration for these NP analogs. Conversely,

transient exposure to TGF-β3 altered construct biochemical and mechanical properties, depending on the dose and duration of exposure. DNA content for most groups decreased with time, with the lowest levels found with the lower doses and shorter exposure times (not shown). Alternatively, GAG content significantly increased with time in every condition. Interestingly, several groups (i.e., 50-7d, 100-3d and 100-7d) produced GAG levels comparable to that of the control group (10-cont) (Fig.4B). Collagen content at week three and six appeared independent TGF-β3 (not shown). Consistent with the increasing GAG content, construct mechanical properties increased with time in culture. By 6 weeks, the equilibrium modulus of the 100-7d grouped reached ~300 kPa, a level matching that of the 10-cont control group (p=0.169). Of note, it was necessary for this very high dose to be present for a full week to exert its effect, as the 100-3d group achieved much lower properties. The intermediate dose applied over 1 week (50-7d) also provided competitive mechanical properties compared to continual exposure or a single dose at the highest level (100-3d) (Fig.4C). Histological evaluation showed that minimal PG was deposited with low doses of TGF-β3 applied for short periods of time. Conversely, very short exposures (one week or less) at higher doses produced staining comparable to that seen for continuous exposure at the lower doses (Fig. 5).

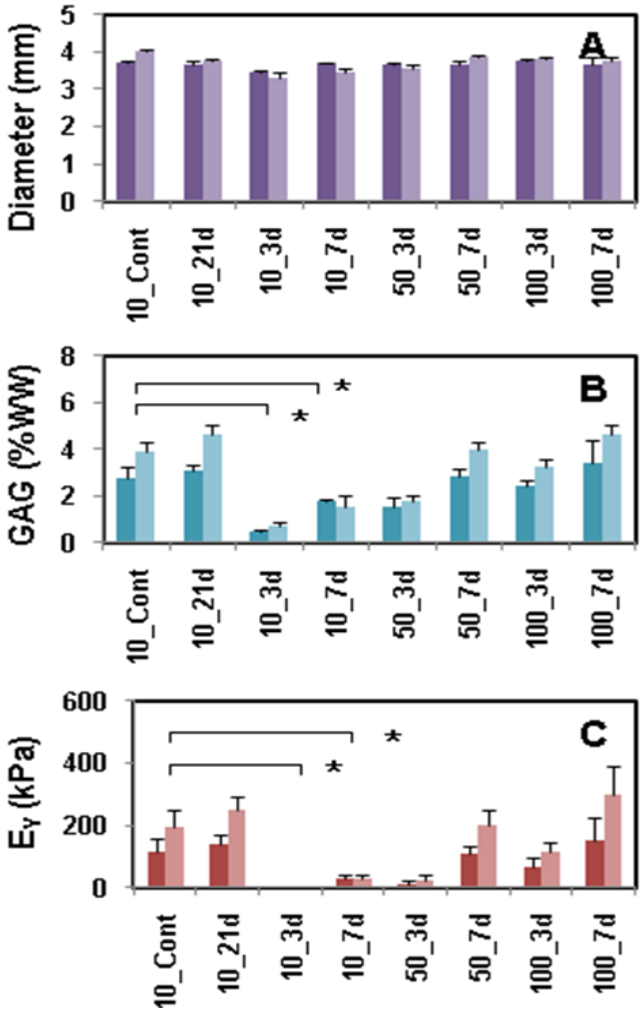

Figure 4: Biochemical and mechanical analysis. (A) Diameter (mm), (B) GAG (% wet weight, ww), (C) Equilibrium Modulus (kPa); N=5/group, Darker = 3w, Lighter = 6w, *p<0.05).

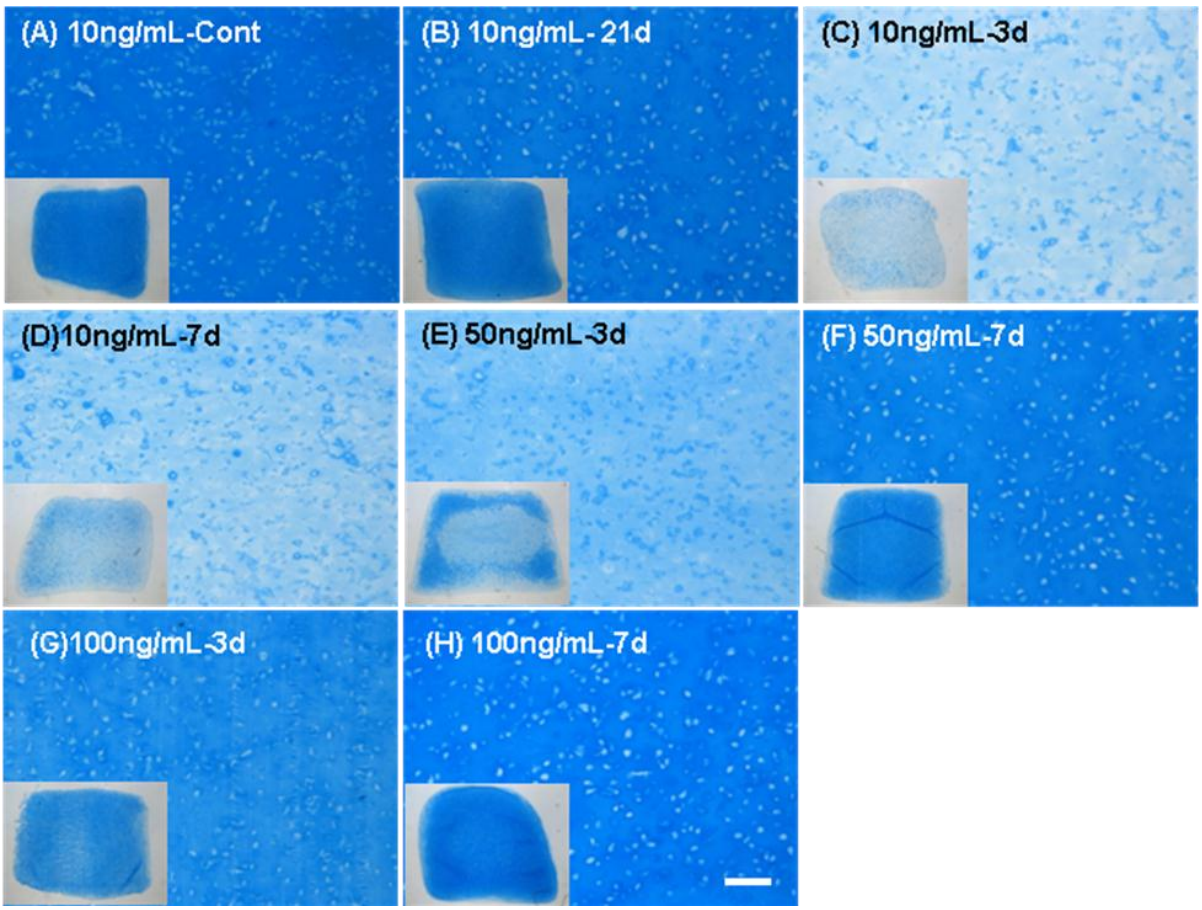

Figure 5: PG staining of constructs at 6 weeks after transient exposure to TGF-ß3 (inset: whole construct). Scale bar = 100 microns.

These findings show a significant impact of TGF-β3 dose and duration of exposure on the functional maturation of MSC-laden HA hydrogels for NP engineering. Our results show that several dose and duration combinations can produce functional NP-like materials, and in some cases do so to a greater extent than continual exposure to lower concentrations. For example, in the most auspicious condition (100 ng/mL, exposure for 7 days), constructs increased in mechanical properties and biochemical content, reaching an equilibrium modulus of >300 kPa and ~5% GAG (per wet weight) after just 6 weeks. These mechanical properties were as high as those in the continual exposure group. This finding confirms that a robust and persistent NP-like state can be induced with very short exposure durations to this morphogen. Our findings point to the translational potential of this system, where a finite (but large) dose of TGF need only be delivered for a short period of time before implantation. Ongoing work in this area is exploring the maturation of this NP conjoined with the AF as described above, and under these growth factor and dosing regimens. Additional ongoing studies are challenging this constructs with serum after the differentiation has occurred, and will set the stage for our our current transition to Aim 2.

Finally, to assess the consequences of implanting engineered constructs into an inflammatory environment, we have performed in vitro studies to determine the response of engineered NP constructs to the presence of the inflammatory cytokine interleukin-1β. NP cells were cultured for 49 days in agarose in a chemically defined media containing TGF-β3 to induce functional

matrix deposition. Constructs were then treated with 10ng/ml IL-1β for 3 days, and effects on biomechanical properties, extracellular matrix composition and mRNA levels were quantified. IL-1β treatment resulted in upregulation of ADAMTS4 and INOS mRNA, and decreased GAG and modulus (Figure 6). These results indicate that DAPS may require enhanced functional properties to effectively persist and integrate in an inflamed disc space. This work has been accepted for publication (pending minor revisions) in European Cells and Materials journal.

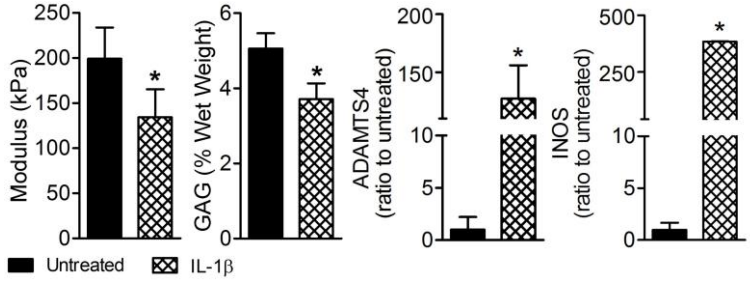

Figure 6: NP constructs respond to inflammatory stimulus. GAG content, modulus, and ADAMTS4 and INOS mRNA. *p<0.05 vs. untreated.

Based on the results of these studies, all portions of Aim 1 have thus been completed or are currently in culture per our original statement of work.

Original Aim 2: Implant the tissue engineered DAPS in a subcutaneous environment after varying period of in vitro pre-culture and evaluate maturation using the same assays and evaluation criteria as in Aim 1.

In preparation for the initiation of work related to Aim 2, we have submitted ACORP protocols regarding DAPS implantation in the subcutaneous space. We have also (using other funds), fully developed this in vivo evaluation method at the VA, with a particular focus on improving scaffolds for the AF region (improving cell infiltration, providing drug delivering capacity). An amendment to our ongoing ACORP is under consideration for the implantation of our MSC-based DAPS, and the long term portions of these studies have been initiated in culture. We anticipate that significant progress will be made on this Aim in the coming year.

Original Aim 3: Implant the tissue engineered DAPS in situ using a rat tail disc replacement model. Evaluate DAPS maturation and in situ integration under static conditions and with resumption of mechanical loading. The engineered DAPS will be evaluated under two fixation conditions: an Immobilization group and Compression group, which is loaded axially with 0.5X body weight. Both of these fixation conditions will be applied for either short duration (8 weeks, followed by release and an additional 8 weeks normal loading) or held for the entire study duration (16 weeks). Structure, mechanics, and composition of the functional DAPS and its interface will be evaluated at 2, 8, and 16 weeks. Both fixation conditions will be compared against a sham control in which all surgery and fixation conditions are the same, but without disc removal or DAPS placement.

This Aim represents the culmination of our project, and will be substantially completed in the 2nd and 3rd years of funding, however, we have made significant accomplishments in the last 12 months. First and foremost, we have obtained ACORP approval for DAPS implantation in the rat disc space, and have piloted surgery (with a few acellular DAPS) to better equip ourselves for

the eventual implantation of the fully matured versions developed in Aims 1 and 2. These advances are detailed below.

We have developed and validated the surgical technique for implantation of the tissue engineered disc into the rat caudal disc space. The surgical technique was designed to be minimally invasive in order to prevent vascular damage and promote rapid healing. Three surgical groups were evaluated: sham, discectomy and implantation of acellular disc constructs. Healing and integration were evaluated at time points up to one month using histology and microCT. At one week, histological evaluation showed the construct well placed in the disc space (Figure 7A), with lamellar architecture characteristic of the native tissue. At subsequent time points however, histology showed migration of the construct out of the disc space, and microCT (Figure 7B) suggested the disc space had subsequently collapsed. To overcome this problem, we have designed and implemented a novel internal fixation device consisting of a miniature intervertebral stainless steel plate, mounted dorsally over the disc space using bone screws. This is designed to enhance retention of the construct in the disc space and stabilize the joint during healing. This bone plate has been validated in a cadaver model (Figure 7C) and will be used for subsequent surgeries.

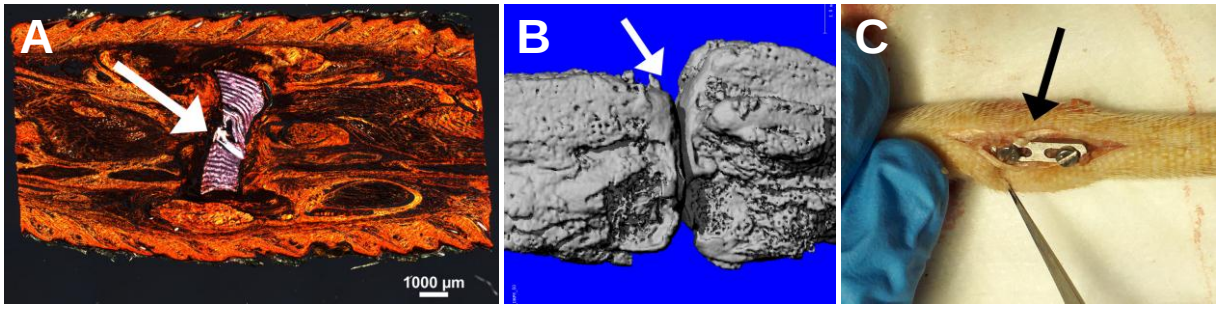

Figure 7: A. Histology showing acellular disc implant (arrow) at 1 week. B. MicroCT showing collapsed disc space (arrow) at 4 weeks. C. Novel bone plate (arrow) to stabilize joint and retain implant in disc space

Key Research Accomplishments:

According to the progress we have made on the Aims outlined above, we have achieved the following Key Research Accomplishments.

- We have established advanced fabrication of DAPS AF regions, and have implemented new culture conditions to improve their maturation with time. (Aim 1)
- We have demonstrated that improvement of nutrient supply, via orbital shaking, improves the DAPS maturation process, suggesting that motion and flows encountered in vivo might improve maturation of constructs. (Aim 1)
- We have constructed and implemented a novel micro-torsion device and shown that DAPS improve in torsional properties with time in culture and shaking. (Aim 1)
- We have determined, with a focus on the NP region, that a short period of exposure to TGF at a high dose is equal to or better than long term exposure to this molecule. This sets the stage for the implementation of this dosing scheme for DAPS in Aim 2, with subcutaneous implantation. (Aim 1)
- We have perfected our techniques for subcutaneous implantation (via other funding mechanisms, in support of Aim 2 being initiated).

- We have more fully developed and validated the rat tail model of disc implantation (Aim 3), including permission from relevant animal care and use committees. We have carried out pilot implantation studies to develop the model, and imaged the DAPS in situ in the disc space. (Aim 3)
- We have developed and validated a minimally invasive surgical technique for implantation of our engineered disc. (Aim 3)
- We have successfully performed in implantation of acellular engineered discs. (Aim 3)
- We have designed and implemented a novel internal fixation device to enhance retention of the engineered disc and stabilize the joint during healing. (Aim 3)

Reportable Outcomes:

Our team has published two abstracts in our first year of this project, and have one paper in press (pending minor revisions) as is detailed above. We anticipate several additional abstract submissions and one additional manuscript in the coming year. Further, portions of this work have been presented at the Philadelphia Spine Society meeting. Funding through this award has supported one doctoral student in mechanical engineering at the University of Pennsylvania as well as several postdoctoral fellows.

Conclusions:

Our team has made marked progress in the formation of DAPS into angle ply structures and optimization of the in vitro growth of both the AF and NP regions. We have discovered important new methods to further this growth process, including a novel TGF dose and exposure regime that generates a functional NP like material. We have validated a rat tail model for the implantation of our DAPS construct, and have designed novel fixation devices for protecting the DAPS post implantation. All methods, materials, and approvals are in place for our team to continue to make important progress in disc tissue engineering over the next year. In summary, we have successfully developed and validated our preclinical animal model, and are poised to implant cellularized, functional mature engineered disc constructs.

References/Publications:

1. Kim M, Erickson IE, Chowdhury M, Pleshko N, Mauck RL, “Transient exposure to TGF-β3 improves the functional properties of MSC-laden hyaluronic acid hydrogels,” *Journal of the Mechanical Behavior of Biomedical Materials*, in revision.
2. Minwook Kim, Isaac E. Erickson, Jason A. Burdick, and Robert L. Mauck**,** “Transient exposure to TGF-ß3 improves the functional properties of MSC-seeded photocrosslinked hyaluronic acid hydrogels,” ASME Summer Bioengineering Conference, Nemacolin Woods, PA, June 2011, poster presentation.
3. Kluge, JA; Martin, JT; Nerurkar, NL; Amaniera, FA; Pampati, RA; Elliott, DM; Mauck R L, “Functional Enhancement of Disc-Like Angle-Ply Structures via Dynamic Culture”, Transactions of the 2011 Orthopaedic Research Society Meeting, Long Beach, California, January 2011, poster presentation.
4. Smith LJ, Nerurkar NL, Cortes DH, Chiaro JA, Horava SD, Hebela NM, Mauck RL, Dodge GR, Elliott DM **“**Nucleus pulposus cells synthesize a functional extracellular matrix and respond to inflammatory cytokine treatment following long term agarose culture”, *European Cells and Materials,* in revision

Appendices: N/A